REMARKS

Status

Claims 66-68, 73-89 are pending in the application. Claims 66, 81 and 85 have been amended. Support for the amendments can be found at page 5, line 17 (Claims 66, 81 and 85) and page 71, lines 11-35 (Claim 66). Accordingly, no prohibited new matter has been added. Entry of the amendments and allowance of the claims is respectfully requested.

Rejection Under 35 U.S.C. §102(e)

Claims 66-68, 73-75, 81-82, 85, and 87-89 stand rejected under 35 U.S.C. §102(e) as allegedly being anticipated by U.S. Patent No. 5,786,168. Applicants respectfully traverse the rejection for the reasons given below.

The Office Action alleges that the instant application teaches a MIF amino acid sequence (i.e., SEQ ID NO:5) which is identical to a sequence recited in the '168 patent (i.e., SEQ ID NO: 38). Thus, by virtue of the alleged identical amino acid sequences, the Action infers that an antibody binding to the GIF protein of the '168 patent will also bind to the MIF as recited in the instant application, and as such, intuitively, the antibody of the '168 patent anticipates the antibody as claimed in the instant methods.

Further, as recited in the Action:

> "Unless applicant can demonstrate that functional differences between MIF and GIF result in the generation of antibodies made against GIF would not specifically bind MIF, they would, because as claimed the antigens the antibodies specifically bind to are structurally identical."

Respectfully, Applicants wish to offer as evidence an article by Mikayama, et al. (Proc Natl Acad Sci USA, 1993, 90:10056-10060) which demonstrates the differences as requested by

the Action (Exhibit A). Please note, that inventors of the '169 patent are also co-authors of the Mikayama et al. (1993) article.

Mikayama et al. (1993) demonstrate the molecular cloning and functional characterization of GIF. From their observations they recorded the following:

> "Since GIF cDNA has high homology to MIF cDNA (18[1]), we determined MIF activity of recombinant GIF. Culture supernatant of COS-1 cells cotransfected with pro-CT-hGIF cDNA and furin cDNA was fractionated on $388F_1$-Affi-Gel[2], and both the effluent and acid eluate fractions were assessed for MIF activity and GIF activity. Neither the effluent nor eluate fraction had MIF activity, although GIF bioactivity was detected in a 1:100 dilution of the eluate fraction. In the same assay, supernatant of COS-1 cells transfected with MIF cDNA showed MIF activity at the final dilution of 1:10, but no GIF activity was detected in a 1:4 dilution of the supernatant." Mikayama et al. (1993), at page 10059, column 2, paragraph 3.

At the onset, please note that the sequence recited in Weiser et al. (1989) was shown to be incorrect by Paralkar and Wistow (1994).[3] As was pointed out in Watarai et al. (2000), the one base difference between GIF and MIF cDNA as identified by Weiser et al. (1989) is due to an error in sequence[4] and "[t]hus, it appears that GIF and MIF share an identical gene . . .".[5] Therefore, the MIF of the instant application is identical to the MIF used in Mikayama et al. (1993).

Further, it is evident that while the genes are structurally identical, GIF and MIF proteins have separate and distinct biological functions which are stereo-selective because separate

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[1] Weiser et al., Proc Natl Acad Sci USA, 1989; 86:7522-7526.

[2] $388F_1$ is a mAb against hybridoma-derived GIF.

[3] Paralkar and Wistow, Genomics, 1994, 19:48-51. See also, Watarai et al., Proc Natl Acad Sci USA, 2000, 97:13251-13256 at 13251, column 1, first paragraph following the Abstract. A courtesy copy of which has been provided for your convenience (Exhibit B). Please note that '168 co-inventor Kimishige Ishizaka is also a co-author of this article.

[4] See, also, instant application at page 66, line to page 67, line 4.

[5] Watarai et al. (2000), at page 13251, column 1, first paragraph following the Abstract.

cognate receptors are responsible for each distinct response.[6] This suggests configuration differences (e.g., separate and distinct surface exposed domains/epitopes).

Mikayama et al. (1993) go on to show:

> "High amino acid sequence homology between hGIF and MIF suggested that GIF might have MIF activity. However, our experiments showed that affinity-purified recombinant hGIF failed to inhibit migration of human monocytes even at a 20 fold higher concentration than that required for the detection of GIF activity. It was also found that affinity purified mGIF from 231F1 cells, with a GIF titer of 1:50, failed to inhibit the migration of mouse macrophages . . .. In contrast, the supernatant of COS-1 cells transfected with MIF cDNA showed a high MIF activity but did not have GIF activity. Our more recent experiments indicated that **MIF activity in the supernatant failed to be retained in either 388F1-coupled Affi-Gel or polyclonal anti-GIF-coupled Affi-Gel. The results collectively indicate that GIF is distinct from MIF.**"[7] (Emphasis added). At page 10060, column 1, paragraph 3 to column 2, paragraph 1.

Also consider that antibody cross-reactivity was observed for polyclonal antibody binding between human GIF and murine GIF, where there is 90% amino acid identity. (See Mikayama et al. (1993) at page 10060, column 1, second paragraph). **Again, this was not observed for said same polyclonal antibodies and MIF**.

And while it was speculated by Mikayama et al. (1993), at page 10060, that the associated MIF bioactivity of the predicted amino acid was not conclusive because, at that time, MIF had not been affinity purified, the Applicants' present disclosure demonstrates that such activity is ascribed to the (correct) sequence as recited. Thus, while seeming counterintuitive, Mikayama et

[6] See, e.g., Watarai et al. (2000), at page 13252, column 1, third full paragraph, "**Inhibition of High Affinity Binding of GIF Derivatives to GIF Receptor**" section.

[7] Applicants point out that Watarai et al. (2000) have demonstrated that the configurations of GIF/MIF that allow for separate biological activity are due to a post-translational modification of Cys-60 comprising the protein, i.e., cysteinylation (see, e.g., Watarai et al. (2000), at pages 13254-13256). Such flexibility in configuration indicates that certain amino acids are exposed to the solvent face for the cysteinylated conformer, but such amino acids are hidden in the non-cysteinylated conformer. (Watarai et al. (2000), at page 13255).

al. (1993) demonstrate "the generation of antibodies made against GIF" that "would not specifically bind MIF" as required by the Action.

Therefore, as Applicants have demonstrated, via Mikayama et al. (1993), that functional differences between MIF and GIF result in the generation of antibodies made against GIF which do not specifically bind to MIF, the '168 patent does not anticipate the instant invention as claimed.

In view of this evidence, Applicants respectfully request that the rejection be withdrawn.

Rejection Under 35 U.S.C. §112, First Paragraph, Enablement

Claims 68-68, 73-75, 81-82, 85, and 87-89 stand rejected under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the enablement requirement. Applicants, respectfully, traverse the rejection for the reasons given below.

While not acquiescing to the arguments provided in the Office Action, and to expedite prosecution towards allowance, Applicants have amended the claims to more clearly define the invention.

In view of the amendment, Applicants respectfully request that the rejection be withdrawn.

CONCLUSION

All of the stated grounds of rejection have been properly traversed, accommodated, or rendered moot. Applicants, therefore, respectfully request that the Examiner reconsider all presently outstanding rejections and that they be withdrawn. It is believed that a full and complete response has been made to the outstanding Office Action and, as such, the present application is in condition for allowance.

If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to contact Daryl A. Basham (Reg. No. 45,869) at the telephone number listed below.

Respectfully submitted,

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Immunology

Molecular cloning and functional expression of a cDNA encoding glycosylation-inhibiting factor

(immunoregulation/macrophage migration inhibitory factor)

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ABSTRACT By using probes based on partial amino acid sequence of glycosylation-inhibiting factor (GIF) from a mouse T-cell hybridoma, a full-length cDNA encoding mouse GIF was isolated. A cDNA clone encoding human GIF was isolated from cDNA libraries of a GIF-producing human T-cell hybridoma by using mouse GIF cDNA as a probe. The cDNAs encode a putative 12.5-kDa peptide of 115 amino acids. Northern blot analysis demonstrated a single, 0.6-kb transcript. Polyclonal rabbit antibodies against the *Escherichia coli*-derived recombinant 13-kDa peptide bound hybridoma-derived GIF. Although the peptide did not contain a signal peptide sequence, transfection of the cDNA into COS-1 cells resulted in secretion of 13-kDa peptide, but the peptide had substantially less bioactivity than the hybridoma-derived GIF. However, expression of a chimeric cDNA encoding a fusion protein consisting of the N-terminal pro region of calcitonin precursor and human GIF and cotransfection with furin cDNA to allow intracellular cleavage of the fusion protein resulted in secretion of 13-kDa peptide that was comparable to hybridoma-derived GIF in its bioactivity. Both the 13-kDa peptide and GIF bioactivity in the transfected COS-1 supernatant bound to a monoclonal antibody against hybridoma-derived human GIF. These results indicate that the 13-kDa peptide represents recombinant GIF, but posttranslational modification of the peptide is important for generation of the bioactivity. The GIF cDNA had high homology with the cDNA encoding macrophage migration inhibitory factor. However, the recombinant GIF failed to inhibit migration of human monocytes, and recombinant human macrophage migration inhibitory factor did not have GIF bioactivity.

Previous studies on regulation of IgE antibody response in rodents described glycosylation-inhibiting factor (GIF), a lymphokine that is involved in selective formation of IgE-suppressive factor (1). GIF inhibits N-glycosylation of IgE-binding factors (IgE-BFs), and the unglycosylated IgE-BFs then selectively suppress IgE synthesis. Subsequent experiments indicated that GIF facilitated the generation of antigen-specific suppressor T cells both *in vivo* (2) and *in vitro* (3) and provided evidence that GIF is a subunit of antigen-specific suppressor T-cell factors (TsFs) (4). This hypothesis is supported by the fact that the monoclonal antibody (mAb) against lipomodulin, 141-B9, binds not only GIF but also representative TsFs from hapten-specific suppressor T-cell hybridomas (5).

We expected that biochemical characterization and molecular cloning of GIF would help to solve controversial issues regarding antigen-specific TsFs. Mouse GIF (mGIF) was purified to homogeneity from serum-free culture supernatant of a representative GIF-producing T-cell hybridoma, 231F1, by affinity chromatography on 141-B9-coupled immunosorbent (6). Subsequently, GIF-producing human T-cell hybridomas were established, and human GIF (hGIF) from a representative hybridoma, AC5, was identified as a 14-kDa peptide by SDS/PAGE (7). Based on these findings, the present experiments were undertaken to isolate cDNA clones that encode mGIF and hGIF.§

MATERIALS AND METHODS

Purification of GIF. mGIF in serum-free culture supernatant of 231F1 cells was purified by using Affi-Gel 10 (Bio-Rad) coupled to mAb 141-B9 (6) or coupled to the IgG fraction of a rabbit antiserum against recombinant mGIF. Recombinant hGIF was fractionated on Affi-Gel 10 coupled to the anti-hGIF mAb 388F$_1$ (7). Usually, 2–5 mg of a mAb or 10 mg of IgG from rabbit antiserum was coupled to 1 ml of gel. Procedures for the fractionation have been described (6, 7). After recovery of the flow-through fraction, the immunosorbent was washed with 20 column volumes of phosphate-buffered saline (0.05 M phosphate/0.15 M NaCl, pH 7.0), and proteins retained in the column were recovered by elution with 0.1 M glycine·HCl buffer (pH 3.0).

Recombinant mGIF expressed in *Escherichia coli* was purified from inclusion bodies. After disruption of *E. coli* cells, the pellet fraction was extracted with 0.2 M Tris·HCl buffer (pH 8.0) containing 6 M guanidine hydrochloride and 25 mM EDTA, and the extract was fractionated on a Sephacryl S-200 column equilibrated with the same buffer. Fractions containing the 13-kDa peptide, detected by SDS/PAGE, were concentrated and slowly added to a large volume of Tris buffer for refolding of peptides. The sample was then applied to a TSK gel DEAE-5PW column (Toyo Soda, Tokyo) equilibrated with 20 mM Tris·HCl buffer (pH 8.0), and proteins were eluted with a gradient of 0–0.1 M NaCl.

Amino Acid Sequencing. Affinity-purified mGIF was precipitated by 10% (wt/vol) trichloroacetic acid (6), electrophoresed in an SDS/15% polyacrylamide gel under reducing conditions, and then electroblotted to a poly(vinylidene difluoride) (PVDF) membrane. The immobilized 13-kDa protein was reduced and S-carboxymethylated *in situ* (8) and digested with 1 pM *Achromobacter* protease I (Wako Pure Chem, Tokyo) at pH 9.0. Peptides retained on PVDF membranes were subdigested with 2 pM endoproteinase Asp-N

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Abbreviations: GIF, glycosylation-inhibiting factor; mGIF, mouse GIF; hGIF, human GIF; IgE-BF, IgE-binding factor; mAb, monoclonal antibody; TsF, suppressor T-cell factor; MIF, macrophage migration inhibitory factor; pro-CT, pro region of calcitonin precursor; PVDF, poly(vinylidene difluoride).

§The sequences reported in this paper have been deposited in the GenBank data base [accession nos. 10612 (hGIF) and 10613 (mGIF)].

(Boehringer Mannheim) in 100 mM ammonium bicarbonate (pH 7.8) containing 8% acetonitrile. Peptides released from the membrane after each digestion were fractionated by reverse-phase HPLC on a μBondapak C_8 column (particle size, 5 μm; pore size, 300 Å; Waters) equilibrated with 0.05% trifluoroacetic acid as a mobile phase. Peptides were eluted by a linear gradient (0–50%) of 0.02% trifluoroacetic acid in 2-propanol/acetonitrile, 7:3 (vol/vol). Amino acid sequence analysis of each peptide was performed with a gas-phase sequencer (Applied Biosystems, model 470A) with modified programs for microsequencing (9).

Construction of cDNA Library. Total cellular RNA was isolated from 231F1 cells (4) or AC5 cells (7) by using RNAzol (Tel-Test, Friendswood, TX). Poly(A)$^+$ RNA was isolated by using a FastTrack mRNA isolation kit (Invitrogen). cDNA libraries were constructed with a Uni-Zap cDNA synthesis kit (Stratagene). After screening of the cDNA library, selected cDNA clones were sequenced by the standard dideoxy method with the Sequenase kit (United States Biochemical). The DNA sequences were analyzed with MACVECTOR software (International Biotechnologies).

Expression of Recombinant GIF. For bacterial expression of mGIF, *Afl* II and *Bam*HI adaptor sites were ligated at both ends of mGIF cDNA by polymerase chain reaction (PCR), and the cDNA fragment was inserted by ligation into pST811 vector (10) carrying the *trp* promoter and *trpA* terminator. The plasmid was transformed into competent *E. coli* RR1 cells, and the cells carrying plasmid were cultured in M9 broth with glucose (0.8%), amino acids (0.4%), thiamin (10 μg/ml), and ampicillin (50 μg/ml). Cells were harvested 5 hr after the addition of indoleacrylic acid (11).

For the expression of GIF cDNA in COS-1 cells, two different types of plasmids were constructed. In one type, mGIF or hGIF cDNA was ligated into *Bgl* II/*Kpn* I-digested modified SRα vector (12). Since GIF does not appear to have a signal peptide sequence, we constructed another expression system for translation of a fusion protein which consisted of the N-terminal pro region of the human calcitonin precursor (pro-CT) and human GIF. Intracellular cleavage of the fusion protein was mediated by an endoprotease, furin (13), allowing the secretion of mature GIF peptide (14). The cDNA fragment encoding pro-CT was amplified by PCR using a human calcitonin cDNA as the template and oligonucleotide primers tailed with a *Pst* I recognition site (14). The amplified gene was cloned into the SRα vector at the *Pst* I site. To fuse hGIF cDNA to the 3′ end of pro-CT cDNA in frame, the 5′ extension method was applied to hGIF cDNA. The sequence of the 5′ primer was 5′-CCAGATCTAAGCG-GATGCCGATGTTCATCGTAAACACC-3′, which contains a *Bgl* II site (see Fig. 1*B*). The amplified hGIF gene was ligated into *Bgl* II/*Kpn* I-digested SRα vector in which pro-CT cDNA had been inserted. Human furin cDNA was cloned into the SRα vector as described (14). The plasmids were transfected into COS-1 cells either by the DEAE-dextran method or by electroporation. After transfection, cells were incubated overnight in a 1:3 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 (DMEM/F12) containing 10% fetal bovine serum and then were cultured for 1 week in serum-free DMEM/F12 containing bovine insulin (20 μg/ml; Sigma), human transferrin (20 μg/ml; Sigma), 40 μM monoethanolamine, 0.1 μM sodium selenite, and bovine serum albumin (1 mg/ml; Sigma) to recover culture supernatant.

Electrophoresis and Immunoblotting. Affinity-purified GIF preparations were analyzed by SDS/PAGE in a 15% polyacrylamide slab gel under reducing conditions (15), and proteins in the gel were visualized by silver staining (16). An aliquot of a sample was analyzed along with serial 2-fold dilutions of *E. coli*-derived recombinant mGIF of known concentrations, and the concentration of the 13-kDa peptide in the sample was estimated by the intensity of the band in silver staining. Immunoblotting was carried out with the enhanced chemiluminescence (ECL) Western blot detection system (Amersham). Polyclonal rabbit antibodies against recombinant mGIF were affinity-purified by absorption of the IgG fraction of the anti-GIF antiserum with Affi-Gel 10 coupled to *E. coli*-derived mGIF, and proteins retained in the column were eluted with glycine·HCl buffer (pH 3.0). Two to 4 μg/ml of the affinity-purified antibodies was used to detect the GIF band.

Detection of GIF Bioactivity. GIF was detected by its ability to switch the mouse T-cell hybridoma 12H5 cells from the formation of glycosylated IgE-BF to the formation of unglycosylated IgE-BF. Detailed procedures for the assay have been described (3). Briefly, the 12H5 cells were cultured with mouse IgE (10 μg/ml) in the presence or absence of a test sample, and IgE-BF in culture filtrates was fractionated on lentil lectin-Sepharose. When the 12H5 cells were cultured with IgE alone, essentially all IgE-BF formed by the cells bound to lentil lectin-Sepharose and was recovered by elution with methyl α-D-mannoside. When a sufficient amount of GIF was added to the 12H5 cells together with IgE, a majority of the IgE-BF formed by the cells was not retained in the column and was recovered in the effluent fraction (3).

Assay for Macrophage Migration Inhibitory Factor (MIF). Human peripheral blood monocytes were employed as indicator cells in an agarose-droplet assay system (17). The assay was set up in triplicate or quadruplicate together with serial dilutions of a supernatant of COS-1 cells transfected with MIF cDNA (18) as a positive control. The area of migration was calculated by the following formula: migration = (diameter of total area/diameter of agarose droplet)^2 − 1. Percent inhibition = 100 − [(average migration of test sample/average migration of negative control) × 100]. In this assay, inhibition of ≥20% was considered to be significant (19).

RESULTS

cDNA Cloning of GIF. mGIF was isolated from culture supernatant of 231F1 cells, and the 13-kDa peptide immobilized on PVDF membrane was employed for determination of partial amino acid sequence. In this experiment, we obtained six different peptides consisting of 12–15 amino acids. Based on the N-terminal amino acid sequence (MPMFIVNTNV-PRASV) and the sequence of one of the fragments (DPCAL-CSLHSIGK), oligonucleotides were synthesized, and PCR was carried out using the two oligonucleotides as primers and single-stranded cDNA of 231F1 cells as the template. A 0.2-kb fragment amplified in the PCR was ligated to pCR1000 vector for subsequent cloning and DNA sequencing. After the nucleotide sequence was confirmed, the 0.20-kb fragment was used to screen a cDNA library from 231Fl cells.

Seven cDNA clones were isolated after screening of 0.5 × 10^6 independent clones. Since restriction mapping of all of the cDNA clones showed a single pattern, the longest clone, with an insert of 0.6 kb, was chosen for DNA sequencing. The nucleotide sequence and deduced amino acid sequence of mGIF are shown in Fig. 1*A*. The largest open reading frame encodes 115 amino acids and the predicted amino acid sequence contained all six peptides obtained by Edman degradation of purified mGIF. The calculated size of the GIF protein is 12.5 kDa, which is in good agreement with that of purified mGIF (6). The nucleotide sequence flanking the first methionine codon favors the translation initiation rule (21). The amino acid sequence downstream from this methionine has a perfect match to that of the N-terminal sequence of the purified GIF, suggesting that GIF has no signal peptide sequence.

Since high homology was expected between mGIF and hGIF, a cDNA library constructed from mRNA of a human

A

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GGCACGACGTCAGGTCCCTGGCTTGGGTCACACCGCGCTTTGTACCGTCCTCCGGTCCAC
-------~AGGCACGTAGCTCAGC--CGGCGCGGCGCG-GC--CTGTG----T-CG-GGG

                                                          13
                      M  P  M  F  I  V  N  T  N  V  P  R  A
GCTCGCAGTCTCTCCGCCACCATGCCTATGTTCATCGTGAACACCAATGTTCCCCGCGCC
T---CTG---CT--T----T------G-----------A--------C--G---------

                                                          33
 S  V  P  E  G  F  L  S  E  L  T  Q  Q  L  A  Q  A  T  G  K
TCCGTGCCAGAGGGGTTTCTGTCGGAGCTCACCCAGCAGCTGGCGCAGGCCACCGGCAAG
--------G--C-----C--C--C------------------------------------
          D
                                                          53
 P  A  Q  Y  I  A  V  H  V  V  P  D  Q  L  M  T  F  S  G  T
CCCGCACAGTACATCGCAGTGCACGTGGTCCCGGACCAGCTCATGACTTTTAGCGGCACG
---C-C-----------G-----------------------------G-C--CG----T-C
    P                                          A     G     S

                                                          73
 N  D  P  C  A  L  C  S  L  H  S  I  G  K  I  G  G  A  Q  N
AACGATCCCTGCGCCCTCTGCAGCCTGCACAGCATCGGCAAGATCGGTGGTGCCCAGAAC
-G---G--G-----G--------------------------------C--C--G------
 S  E
                                                          93
 R  N  Y  S  K  L  L  C  G  L  L  S  D  R  L  H  I  S  P  D
CGCAACTACAGTAAGCTGCTGTGTGGCCTGCTGTCCGATCGCCTGCACATCAGCCCGGAC
---TC------C---------C----------G----G-------G-------------
    S                             A  E        R
                                                         113
 R  V  Y  I  N  Y  Y  D  M  N  A  A  N  V  G  W  N  G  S  T
CGGGTCTACATCAACTATTACGACATGAACGCTGCCAACGTGGGCTGGAACGGTTCCACC
A----------------------------G-----T------------AAC------
                                     ↑                 N
 F  A
TTCGCTTGAGTCCTGGCCCCACTTACCTGCACCGCTGTTCTTTGAGCCTCGCCTCTCCAC
-----C-A--AG-C-AAGGG--CC--GCTGT-T--GC-GGC-CC-C--GG-AAC-CG-CG

GTAGTGTTCTGTGTTTATCCACCGGTAGCGATGCCCACCTTCCAGCCGGGAGAAATAAAT
CAC-CTG-G-TCTAGGCC-GC--ACCCCAACCTT-TGGTGGGG--AAATA-ACGG-TT-G

GGTTTATAAGAGACCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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B

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                N-terminal pro-region of
                |-------------------------------
                M  G  F  Q  K  F
        (Pst-1) ATGGGCTTCCAAAAGTTC------

     Calcitonin precursor |        human  GIF
  ------ ------------------|-------------------------------|
         P  R  S  K  R ↓M  P  M  F           T  F  A  *
     ----CCCAGATCTAAGCGGATGCCGATGTTC ------ ACCTTCGCCTAA (KpnI)
            BglII
```

FIG. 1. (*A*) Structure of GIF cDNA clones. The second line shows the full-length nucleotide sequence of a mGIF cDNA clone, and the first line shows the predicted amino acid sequence of mGIF. The third and fourth lines show the nucleotide and amino acid sequences, respectively, of a human cDNA clone. Only differences from the mGIF sequence are shown. Arrow indicates the only difference between hGIF cDNA and human MIF cDNA, which has AGT (serine). Six peptides obtained from 231F1-derived 13-kDa GIF corresponded to amino acids 1–15, 13–26, 55–67, 67–78, 86–100, and 101–115 in the deduced amino acid sequence of mGIF. (*B*) Nucleotide sequence of the insert encoding a fusion of pro-CT with hGIF. Deduced amino acid sequence is shown above the nucleotide sequence. The recognition motif for furin is Arg-Xaa-Lys-Arg (20). The cleavage site is shown by an arrow. The insert was ligated into SRα vector through *Pst* I and *Kpn* I sites, as indicated in parentheses.

GIF-producing hybridoma, AC5, was screened with the mGIF cDNA as a probe. Among 27 clones hybridized, 4 clones having a 0.5-kb insert were sequenced, and the structure was compared with that of mGIF (Fig. 1*A*). The hGIF and mGIF sequences were 80% identical at the whole cDNA level, 89% identical in the putative coding region, and 90% identical at the amino acid level. The sequence of the coding region of hGIF cDNA was almost identical to the sequence of human MIF cDNA (18). The only difference is that amino acid 106 of MIF is serine, whereas the corresponding residue of hGIF is asparagine (see Fig. 1*A*).

The expression and the size of transcripts that hybridized to the GIF cDNA were examined by Northern analysis. Surprisingly, GIF mRNA was detected in all of the mouse cell line cells tested—231F1, CTLL-2, BW5147, A20.3, and NIH 3T3 (fibroblast). Various human cell line cells such as AC5, CEM, RPMI8866, WI-38 (embryonic fibroblast), and PC3 (prostate carcinoma cells) also contained mRNA which hybridized to the hGIF cDNA. Only a single transcript of 0.6 kb was observed in mouse or human cell line (Fig. 2). Northern blotting of RNAs from mouse tissues showed a dominant expression of GIF mRNA in brain, liver, and kidney. Since the size of the transcript is close to the size of the GIF cDNA (584 bp), it is likely that the mGIF and hGIF clones isolated represent full-length cDNAs of GIF.

Isolation of Hybridoma-Derived GIF by Use of Antibodies Against Recombinant 13-kDa Peptide. If the cDNA clones actually encode GIF, one may expect that antibodies against recombinant 13-kDa peptide will bind GIF from T-cell hybridomas. To test this possibility, rabbit antibodies against the *E. coli*-derived 13-kDa peptide were obtained. The purity of the recombinant mouse peptide employed for immunization was >95% as determined by SDS/PAGE (Fig. 3*A*), and the N-terminal amino acid sequence of the peptide corresponded to that predicted from the nucleotide sequence of the cDNA. Rabbits were immunized by an intramuscular injection of 100 μg of peptide included in complete Freund's adjuvant, and the antiserum was obtained after five booster injections. Since IgG at 2–4 μg/ml in the antiserum was adequate for detection of the recombinant 13-kDa peptide by Western blotting, culture filtrate of the 231F1 cells was fractionated on Affi-Gel 10 coupled with the IgG fraction of the antiserum. GIF activity was not detectable in the flow-through fraction, and >80% of the bioactivity in the culture filtrate was recovered in the eluate fraction, which gave a 13-kDa band upon SDS/PAGE (Fig. 3*A*). Estimation of the concentration of the 13-kDa peptide in the eluate fraction indicated that a peptide concentration of 5 ng/ml was sufficient for detection of GIF bioactivity (Table 1). Similar experiments were carried out with culture supernatants of a GIF-producing human T-cell hybridoma. Essentially all GIF activity in the supernatant bound to the anti-GIF Affi-Gel and was recovered by acid elution. The minimum concentration

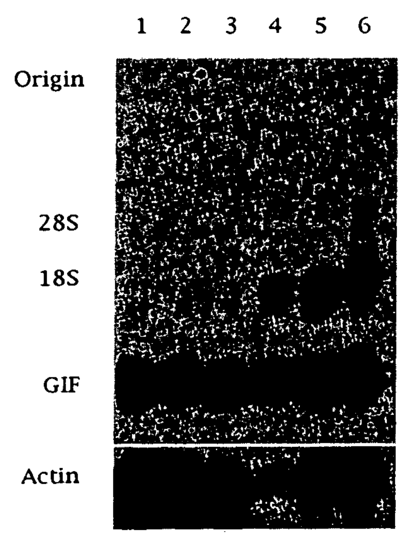

FIG. 2. Expression of the 0.6-kb GIF transcript. Lanes: 1, 231F1; 2, BW5147 (thymoma); 3, CTLL-2 (cytotoxic T-cell line); 4, A20.3 (B-cell line); 5, PT-18 (mouse mast cell line); 6, NIH 3T3 (fibroblast line). Samples (10 μg) of cellular RNA were electrophoresed in formaldehyde/3% agarose gel and blotted to a charged nylon membrane. After probing with the ^{32}P-labeled mGIF cDNA, the same membrane was stripped and hybridized with a PCR-amplified β-actin cDNA labeled with ^{32}P under the same conditions. Conditions for hybridization were 50% formaldehyde/5× standard saline citrate (SSC)/1× Denhardt's solution/0.5% SDS at 42°C, followed by subsequent washing with 2× SSC/0.1% SDS at 25°C and 0.5× SSC/0.1% SDS at 65°C. Positions of 28S and 18S rRNA are shown.

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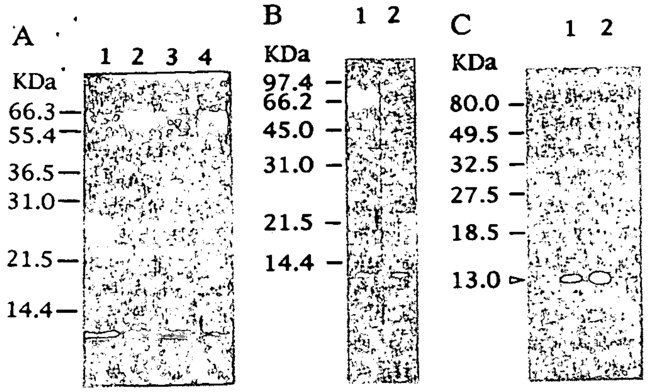

FIG. 3. Identification of hybridoma-derived GIF and recombinant GIF by SDS/PAGE. (*A*) Comparisons among *E. coli*-derived mGIF, 231F1-derived mGIF, and GIF from COS-1 cells transfected with mGIF cDNA. *E. coli*-derived mGIF, isolated from inclusion bodies, was applied to lane 1. The 231F1-derived GIF (lane 2) and COS-1-derived GIF (lane 3) were purified by using Affi-Gel 10 coupled to polyclonal antibodies against recombinant mGIF. Supernatant of COS-1 cells transfected with SRα vector alone was absorbed with the same immunosorbent, and the acid eluate fraction was applied to lane 4. Peptides were detected by silver staining. (*B* and *C*) Recombinant hGIF expressed in COS-1 cells. Mature 13-kDa GIF was detected by silver staining (*B*) and by Western blotting (*C*). hGIF cDNA ligated into SRα vector was transfected into COS-1 cells, and recombinant GIF in COS-1 supernatant was purified on 388F$_1$-Affi-Gel (lane 1). hGIF was expressed by cotransfection of a chimeric cDNA encoding pro-CT–hGIF fusion protein and furin cDNA and was purified by the same procedure (lane 2).

of the 13-kDa peptide required for the detection of GIF activity in the eluate fraction was estimated to be 10 ng/ml (Table 1).

Production of Bioactive Recombinant GIF in COS-1 Monkey Cells. The mGIF cDNA was ligated into a modified SRα vector, and the plasmid was transfected into COS-1 cells. Culture supernatant of the transfected cells contained GIF bioactivity and the 13-kDa peptide, which was detected by Western blotting using polyclonal anti-GIF antibodies. Supernatants from GIF-transfected COS-1 cells and from mock-transfected cells were fractionated with the anti-GIF coupled to Affi-Gel. Essentially all GIF bioactivity in the supernatant of GIF-transfected cells bound to the immunosorbent and was recovered by acid elution, whereas the activity was not detectable in the acid eluate fraction of the supernatant from mock transfectants. The 13-kDa peptide was detected in the eluate of GIF-transfected COS supernatant but barely detectable in the fraction from mock-transfected cells (Fig. 3*A*). The hGIF cDNA was expressed in COS-1 cells using the same vector, and the supernatants were fractionated on 388F$_1$-Affi-Gel. As expected, all GIF bioactivity in the culture supernatant of GIF-transfected cells bound to the immunosorbent and was recovered by acid elution. The acid eluate fraction gave a 13-kDa band upon SDS/PAGE (Fig. 3*B*), and polyclonal anti-GIF antibodies bound to the band on a Western blot (Fig. 3*C*). These results collectively indicate that the 13-kDa peptide formed by transfected cells has GIF bioactivity. However, titration of GIF bioactivity in the affinity-purified recombinant mGIF and hGIF and estimation of the concentration of 13-kDa peptide in the preparations by SDS/PAGE indicated that the concentration of recombinant GIF required for the detection of GIF activity was 150–250 ng/ml (Table 1).

Quantitative difference in the biologic activities between the hybridoma-derived GIF and recombinant GIF suggested to us the possibility that bioactivity of the 13-kDa peptide may depend on posttranslational modification of the peptide. Since GIF does not have a signal peptide (Fig. 1*A*), we applied a device for the secretion of a recombinant truncated peptide via the constitutive pathway (14). Our approach was to fuse the cDNA fragment encoding human pro-CT with GIF-cDNA for the expression of a fusion protein in COS-1 cells and to utilize furin for intracellular cleavage of the fusion protein and subsequent secretion of the mature GIF. The nucleotide sequence of the insert encoding the fusion protein and the predicted amino acid sequence around the cleavage site are shown in Fig. 1*B*. Indeed, cotransfection of the cDNA encoding the fusion protein and furin cDNA resulted in secretion of the 13-kDa GIF. Bioactivity of the supernatant was 5–10 times higher than that of COS-1 cells transfected with hGIF cDNA. The supernatant contained the 13-kDa peptide, which could be affinity-purified by 388F$_1$-Affi-Gel, and the peptide band in SDS/PAGE bound polyclonal anti-GIF on a Western blot (Fig. 3 *B* and *C*). As expected, essentially all GIF bioactivity in the culture supernatant was recovered in the acid eluate fraction from 388F$_1$-Affi-Gel. Further fractionation of the eluate on polyclonal anti-GIF coupled Affi-Gel indicated that both the 13-kDa peptide and the GIF bioactivity in the fraction bound to the antibodies and were recovered by acid elution. It was also found that GIF activity in the original culture supernatant bound to anti-lipomodulin (141-B9)-coupled Affi-Gel. Titration of GIF bioactivity in the affinity-purified GIF preparations showed that the specific bioactivity of recombinant hGIF obtained by this method was comparable to that of hybridoma-derived GIF (Table 1).

Since the GIF cDNA has high homology to MIF cDNA (18), we determined MIF activity of recombinant GIF. Culture supernatant of COS-1 cells cotransfected with pro-CT–hGIF cDNA and furin cDNA was fractionated on 388F$_1$-Affi-Gel, and both the effluent and acid eluate fractions were assessed for MIF activity and GIF activity. Neither the effluent nor eluate fraction had MIF activity, although GIF bioactivity was detected in a 1:100 dilution of the eluate fraction. In the same assay, supernatant of COS-1 cells transfected with MIF cDNA showed MIF activity at the final dilution of 1:10, but no GIF activity was detected in a 1:4 dilution of the supernatant.

Table 1. Bioactivity of hybridoma-derived GIF and recombinant (r) GIF

Purified GIF	Cell source	Antibody used for purification	13-kDa peptide for GIF activity,* ng/ml
mGIF	231F1	Anti-GIF	5
hGIF	31E9	Anti-GIF	10
rmGIF	COS-1	Anti-GIF	250
rhGIF	COS-1	388F$_1$	150
rhcGIF†	COS-1	388F$_1$	10
		Anti-GIF	5
		141-B9	10

GIF in culture supernatant was purified by using Affi-Gel immunosorbent coupled either to polyclonal anti-recombinant mGIF (anti-GIF) or with mAb against hybridoma-derived GIF (388F$_1$), or monoclonal anti-lipomodulin (141-B9).

*Minimum concentration of the 13-kDa peptide required for the detection of GIF bioactivity. These values were calculated from the concentration of the 13-kDa peptide in an affinity-purified GIF and GIF bioactivity in serial 2-fold dilutions of the purified preparations.

†Recombinant hGIF obtained by cotransfection of a chimeric cDNA encoding a pro-CT–hGIF fusion protein and furin cDNA.

DISCUSSION

In this paper, we describe the molecular cloning of cDNAs coding for mGIF and hGIF. Both cDNA clones contain a single open reading frame of 345 nucleotides which encodes a peptide of 115 amino acids. The predicted amino acid

sequence of mGIF was exactly the same as that of a growth factor-induced delayed early response gene (22), and the nucleotide sequence of hGIF cDNA was identical to that of MIF cDNA (18), except for one base (Fig. 1*A*). The hydrophobicity plot of the amino acid sequence of both mGIF and hGIF revealed that hydrophobic and hydrophilic regions are clearly separated and that the length of each region is 20–25 residues. This finding suggests that GIF is a globular protein and that three-dimensional structure of the molecules may be important for their biologic function.

Important findings from the biologic viewpoint were (*i*) that transfection of the GIF cDNA into COS-1 cells resulted in the secretion of bioactive 13-kDa peptide and (*ii*) that the recombinant GIF bound to both the mAb against hybridoma-derived GIF and anti-lipomodulin, while the polyclonal antibodies against recombinant mouse 13-kDa peptide specifically bound hybridoma-derived mGIF and hGIF. When one considers the 90% identity in amino acid sequence between mGIF and hGIF, crossreaction of the antibodies with hGIF is reasonable. Furthermore, bioactivity of the recombinant 13-kDa peptide which was obtained by cotransfection of the chimeric gene encoding a pro-CT-hGIF fusion protein and furin cDNA was comparable to that of hybridoma-derived GIF. These findings collectively indicate that the recombinant 13-kDa peptide actually represents GIF. However, the 13-kDa peptide obtained by transfection of either mGIF cDNA or hGIF cDNA alone was 10- to 30-fold less active than the hybridoma-derived GIF. Since GIF does not have a signal peptide, one may predict that the recombinant 13-kDa peptide synthesized in this system will not go through the endoplasmic reticulum. Mechanisms underlying the secretion of soluble factors without signal peptides—interleukins 1α and 1β—remain unclear (23). Nevertheless, the pro-CT-GIF fusion protein synthesized in COS-1 cells will go through the endoplasmic reticulum and Golgi apparatus, where the fusion protein is cleaved by the furin coexpressed in these cells (13). One may speculate that posttranslational modification of the 13-kDa peptide—e.g., proper folding of the peptide, intrachain disulfide formation, or phosphorylation—is important for the generation of GIF bioactivity. This idea may explain the fact that essentially all cell line cells and mouse tissues contained mRNA for GIF (Fig. 2), whereas the major cell source of bioactive GIF is limited to certain subsets of lymphocytes (1). It has been shown that Lyt-2^+ splenic T lymphocytes and antigen-specific murine suppressor T hybridomas secreted bioactive GIF but that helper T-cell clones and hybridomas did not. Our recent experiments showed that the murine $CD4^+$ T-cell hybridoma 12H5 and human $CD4^+$ T-cell line CEM secreted the 13-kDa peptide which reacted with polyclonal anti-GIF; however, even at 0.2–1.0 μg/ml, the peptide from these cells did not exert GIF bioactivity. One might speculate that the 13-kDa peptide translated in suppressor T cells is modified for the secretion of bioactive GIF, while similar posttranslational modification of the peptide does not occur in helper T cells. Elucidation of the mechanisms for the formation and secretion of bioactive 13-kDa peptide by suppressor T cells requires further studies.

High amino acid sequence homology between hGIF and MIF suggested that GIF might have MIF activity. However, our experiments showed that affinity-purified recombinant hGIF failed to inhibit migration of human monocytes even at a 20-fold higher concentration than that required for the detection of GIF activity. It was also found that affinity-purified mGIF from 231F1 cells, with a GIF titer of 1:50, failed to inhibit the migration of mouse macrophages (results not shown). In contrast, the supernatant of COS-1 cells transfected with MIF cDNA showed a high MIF activity but did not have GIF activity. Our more recent experiments indicated that MIF activity in the supernatant failed to be retained in either $388F_1$-coupled Affi-Gel or polyclonal anti-GIF-coupled Affi-Gel. The results collectively indicate that GIF is distinct from MIF. Since recombinant MIF has not been affinity-purified, it is not conclusive that the 13-kDa peptide of the predicted amino acid sequence has the MIF activity. At present, however, the possibility cannot be excluded that a single amino acid difference between GIF and MIF may account for their biologic activities. Lanakan *et al.* (22) reported that the cDNA probe of a growth factor-induced delayed early response gene, which has exactly the same sequence as our mGIF cDNA, hybridized with a large number of murine and human genomic restriction fragments, suggesting that there is a family of MIF-like genes. The GIF gene appears to belong to this family but is distinct from the MIF gene.

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1. Ishizaka, K. (1984) *Annu. Rev. Immunol.* **2**, 159–182.
2. Akasaki, M., Jardieu, P. & Ishizaka, K. (1986) *J. Immunol.* **136**, 3172–3179.
3. Iwata, M. & Ishizaka, K. (1988) *J. Immunol.* **141**, 3270–3277.
4. Jardieu, P., Akasaki, M. & Ishizaka, K. (1987) *J. Immunol.* **138**, 1494–1501.
5. Steele, J. K., Kuchroo, V. K., Kawasaki, H., Jayaraman, S., Iwata, M., Ishizaka, K. & Dorf, M. E. (1989) *J. Immunol.* **142**, 2213–2220.
6. Tagaya, Y., Mori, A. & Ishizaka, K. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 9117–9121.
7. Thomas, P., Gomi, H., Takeuchi, T., Carini, C., Tagaya, Y. & Ishizaka, K. (1992) *J. Immunol.* **148**, 729–737.
8. Iwamatsu, A. (1992) *Electrophoresis* **13**, 142–147.
9. Iwamatsu, A., Aoyama, H., Dibo, G., Tsunasawa, S. & Sakiyama, F. (1991) *J. Biochem. (Tokyo)* **110**, 151–158.
10. Matsuki, S., Ozawa, T., Nagao, S., Hirata, H., Kanoh, H. & Nozawa, Y. (1990) *Biotechnol. Appl. Biochem.* **12**, 284–291.
11. Nichols, B. P. & Yonofsky, C. (1983) *Methods Enzymol.* **101**, 155–164.
12. Takabe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M. & Arai, N. (1988) *Mol. Cell. Biol.* **8**, 466–472.
13. Wise, R. J., Barr, P. J., Wong, P. A., Kiefer, M. C., Brake, A. J. & Kaufman, R. J. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 9378–9382.
14. Liu, Y.-C., Kawagishi, M., Mikayama, T., Inagaki, Y., Takeuchi, T. & Ohashi, H. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 8957–8961.
15. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
16. Oakley, B. R., Kirsch, D. R. & Morris, N. R. (1980) *Anal. Biochem.* **105**, 361–363.
17. Remold, H. G. & Mendis, A. D. (1985) *Methods Enzymol.* **116**, 379–394.
18. Weiser, W. Y., Temple, P. A., Wtek-Giannott, J. S., Reynold, H. G., Clark, S. C. & David, J. R. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7522–7526.
19. Weiser, W. Y., Greineder, D. K., Reynold, H. G. & David, J. R. (1981) *J. Immunol.* **126**, 1958–1962.
20. Hosaka, M., Nagahama, M., Kim, W.-S., Watanabe, T., Hatsuzawa, K., Ikemizu, K., Murakami, K. & Nakayama, K. (1991) *J. Biol. Chem.* **266**, 12127–12130.
21. Kozak, M. (1983) *Microbiol. Rev.* **47**, 1–45.
22. Lanakan, A., Williams, J. B., Sanders, L. K. & Nathans, D. (1992) *J. Mol. Cell. Biol.* **12**, 3919–3929.
23. Rubartelli, A., Cozzolino, F., Talio, M. & Sitia, R. (1990) *EMBO J.* **9**, 1503–1510.

Posttranslational modification of the glycosylation inhibiting factor (GIF) gene product generates bioactive GIF

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Glycosylation inhibiting factor (GIF) and macrophage migration inhibitory factor (MIF) share an identical structure gene. Here we unravel two steps of posttranslational modifications in GIF/MIF molecules in human suppressor T (Ts) cell hybridomas. Peptide mapping and MS analysis of the affinity-purified GIF from the Ts cells revealed that one modification is cysteinylation at Cys-60, and the other is phosphorylation at Ser-91. Cysteinylated GIF, but not the wild-type GIF/MIF, possessed immunosuppressive effects on the *in vitro* IgE antibody response and had high affinity for GIF receptors on the T helper hybridoma cells. *In vitro* treatment of wild-type recombinant human GIF/MIF with cystine resulted in preferential cysteinylation of Cys-60 in the molecules. The cysteinylated recombinant human GIF and the Ts hybridoma-derived cysteinylated GIF were comparable both in the affinity for the receptors and in the immunosuppressive activity. Polyclonal antibodies specific for a stretch of the amino acid sequence in α2-helix of GIF bound bioactive cysteinylated GIF but failed to bind wild-type GIF/MIF. These results strongly suggest that cysteinylation of Cys-60 and consequent conformational changes in the GIF/MIF molecules are responsible for the generation of GIF bioactivity.

We have previously described glycosylation inhibiting factor (GIF), 13-kDa cytokine, as a product of suppressor T (Ts) cells (1, 2) and a subunit of antigen (Ag)-specific Ts cell factor (3, 4). Repeated injections of partially purified GIF into BDF1 mice resulted in suppression of both IgE and IgG antibody (Ab) responses to ovalbumin (OVA) (5). After molecular cloning of this cytokine, however, we realized that the sequence of the coding region of human GIF cDNA (6) was identical to the sequence of human MIF cDNA (7), except one base. In the human genomes, Paralkar and Wistow (8) identified only one functional MIF-like gene, whose predicted transcript sequence agreed exactly with that of human GIF cDNA, indicating that the one base difference between GIF and MIF cDNA is due to an error in sequencing. Nucleotide sequence of mouse MIF cDNA, described by Bernhagen *et al.* (9), is identical to the sequence of mouse GIF cDNA (6). Thus, it appears that GIF and MIF share an identical gene in both species.

Another unexpected finding was that the GIF/MIF gene is expressed in essentially all murine and human cell line cells examined (6). Many of these cell line cells secreted the 13-kDa peptide that reacted with polyclonal Abs against recombinant human (rh) GIF, however, only the 13-kDa peptide secreted from Ts hybridomas demonstrated GIF bioactivity (10). *Escherichia coli*-derived rhGIF/MIF also lacked GIF bioactivity. It also was found that both the murine Ts hybridomas and the stable transfectant of hGIF cDNA in the Ts hybridoma secreted bioactive GIF, and contained a substantial quantity of inactive GIF in cytosol, and that the amino acid sequence of the cytosolic inactive GIF peptide was identical to that of bioactive homolog in culture supernatants (11). These findings collectively suggested to us the possibility that bioactive GIF is generated by posttranslational modifications of the inactive GIF peptide in Ts cells, and that heterogeneity of GIF bioactivity is due to conformational transition of the same peptide (10).

The hypothesis was supported by the results of subsequent experiments that indicated that *E. coli.*-derived inactive rhGIF could be converted to bioactive derivatives by chemical modification of a single cysteine residue at position 60 (Cys-60) with a sulfhydryl reagent, such as iodoacetate or 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (12). Furthermore, MS analysis of GIF in the culture supernatant and cytosol of the human Ts hybridoma, 31E9 cells, provided direct evidence that GIF protein was posttranslationally modified in the Ts cells (13). Approximately 60% of GIF molecule in the culture supernatant of 31E9 cells had a M_r of 12,429, 12,467, or 12,551, whereas the remaining 40% had a M_r of 12,346, which is identical to the theoretical value calculated from the amino acid sequence. In contrast, inactive cytosolic GIF in the same cells was homogeneous and represented the 12,346 M_r species.

In the present study, attempts were made to identify the biochemical nature of the posttranslational modification of GIF/MIF in Ts cells. The results indicate that cysteinylation of Cys-60 in the GIF sequence occurs in Ts cells and that this modification is responsible for the generation of bioactivity. Evidence is presented that the cysteinylation of Cys-60 induces conformational changes in the GIF molecules.

Materials and Methods

Cell Lines. The human Ts hybridoma 31E9 (6), a subline of human lymphoblastoid cell line CEM(BUC) (14), and the murine T helper (Th) cell hybridoma 12H5 (15) have been described. They were maintained in high glucose DMEM containing 10% FCS and ingredients (15).

Animals. $OVA_{323-339}$-specific T cell receptor (TCR)$\alpha\beta$ transgenic mice on BALB/c background (16) were supplied by Sonoko Habu (Tokai University School of Medicine, Kanagawa, Japan). BALB/c mice were purchased from Nippon Charles River Laboratories, Kanagawa, Japan.

Ags and Abs. Crystalline OVA and BSA were purchased from Sigma. Dinitrophenyl (DNP)-OVA, DNP-BSA, and biotinylated

Abbreviations: GIF, glycosylation inhibiting factor; MIF, macrophage migration inhibitory factor; Ts, suppressor T; Th, T helper; TCR, T cell receptor; rhGIF, recombinant human GIF; OVA, ovalbumin; DNP, dinitrophenyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Ag, antigen; HiTrap, HiTrap *N*-hydroxysuccinimide-activated Sepharose; API, acromobacter protease I.

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DNP-BSA were prepared by the method described (13). $OVA_{323-339}$ peptide (17), HG3 peptide that represents a portion of GIF sequence: Ala-58–Leu–Cys–Ser–Leu–His–Ser–Ile–Gly–Lys–Ile–Gly–Gly–Ala–Gln–Asn–Arg-74; HG3a peptide, Ala-58–Leu–Cys–Ser–Leu–His–Ser–Ile–Gly–Lys–Ile–Gly-69, and HG3b peptide, Ile-65–Gly–Lys–Ile–Gly–Gly–Ala–Gln–Asn–Arg-74 were synthesized in our laboratory by using a model 431A peptide synthesizer (PE Biosystems). A preparation of the mouse IgE anti-DNP mAb H1DNP-ε-26 (18) has been described. Anti-mouse IgE mAb 6HD5 (19) was purchased from Seikagaku Kogyo (Tokyo). Polyclonal anti-hGIF antiserum was prepared as described (13), and the IgG fraction of the serum was obtained by using HiTrap protein G (Amersham Pharmacia). Polyclonal anti-HG3 peptide antiserum was obtained by immunization of rabbits with 100 μg of quadruple-stranded multiple antigenic peptide of HG3 peptide emulsified in complete Freund's adjuvant, followed by eight booster injections of 100 μg of the same immunogen emulsified in incomplete Freund's adjuvant. Anti-HG3 Abs in the antiserum were specifically purified by using HG3 peptide coupled to HiTrap *N*-hydroxysuccinimide-activated Sepharose (HiTrap, Amersham Pharmacia). Five milligrams of the IgG fraction of the polyclonal anti-GIF or 1 mg of specifically purified anti-HG3 Ab was coupled to 5 ml or 1 ml of HiTrap.

Preparation of GIFs. Culture supernatant (1 liter) of the 31E9 cells in serum-free high glucose DMEM was concentrated 40-fold, and the concentrated sample was circulated overnight at 4°C through a 5-ml column of anti-GIF-coupled HiTrap. After washing with 40 column volumes of PBS, proteins retained in the column were eluted with 0.1 M glycine-HCl buffer (pH 2.8). The affinity-purified GIF was further fractionated on a Superdex 75 gel filtration column (1 × 30 cm, Amersham Pharmacia) equilibrated with PBS.

The rhGIF and the mutant, C57A/N106S, in which Cys-57 and Asn-106 in rhGIF were replaced with Ala and Ser, respectively, were expressed in *E. coli* and were purified as described (13). Purified C57A/N106S was radiolabeled with ^{125}I by the method described (20).

SDS/PAGE and Immunoblotting. Affinity-purified GIF preparation was analyzed by SDS/PAGE on 5–15% gradient polyacrylamide gel under reducing condition. Immunoblotting was carried out by using 1 μg/ml of polyclonal anti-GIF or the same concentration of specifically purified anti-HG3 Abs as described (3).

Peptide Map of GIF and MS Analysis. Approximately 1.3 μg (100 pmol) of the affinity-purified GIF or rhGIF in 40 μl of 0.3 M Tris·HCl (pH 6.8) buffer containing 6 M guanidine-HCl was incubated at 60°C for 1 h. After the addition of 5 pmol of acromobacter protease I (API) (Wako Pure Chemical, Osaka) in 40 μl of H_2O, the mixture was incubated at 37°C for 6 h. Then 0.8 pmol of AspN (Boehringer Mannheim) suspended in 160 μl of H_2O was added to the mixture. After 15 h incubation at 37°C, digested peptides were fractionated by semimicro HPLC system (Hitachi, Tokyo) on a super octadecyl silane (ODS) (0.2 × 5 cm, Tosoh, Tokyo) equilibrated with a mixture of 99% (vol/vol) solution A (0.1% TFA) (Nacalai Tesque, Tokyo) and 1% solution B (0.08% TFA/80% acetonitrile). The column was washed with the mobile phase solution for 5 min. The peptides were eluted, at a flow rate 0.2 ml/min from the column with a linear gradient of 1–56% solution B over a period of 55 min. Amino acid sequence of each peptide was determined by using a gas-phase amino acid sequencer model 492 (PE Biosystems). The modified peptides were digested with carboxypeptidase Y (PE Biosystems) in 30 mM ammonium citric acid buffer (pH 6.1) at 25°C for 5 min. Mass spectra of peptides were obtained by the method described (13) using a matrix-assisted laser desorption ionization-time of flight (TOF) mass spectrometer (Voyager Elite DE STR, PE Biosystems).

Cysteinylation of rhGIF. rhGIF (1.3 mg; 100 nmol) in 20 mM sodium phosphate buffer (pH 7.6) containing 0.15 M NaCl was incubated overnight at room temperature with 25 mM L(-)-cystine (Wako Pure Chemical). After removal of excess cystine by gel filtration, GIF proteins in 20 mM sodium acetate buffer (pH 6.0) were applied to a CM-5PW column (0.75 × 7.5 cm, Tosoh) and eluted with a gradient of 0–0.3 M NaCl. The concentration of endotoxin in the final cysteinylated GIF preparations, determined by Limulus ES-II Single Test (Wako Pure Chemical), was <1 ng of lipopolysaccharide/mg of GIF.

***In Vitro* IgE Ab Response.** OVA-specific TCRαβ-transgenic mice-derived splenocytes (4×10^6/well) were suspended in 4 ml of Click's medium (Sigma) supplemented with 10% FCS, 50 μM 2-mercaptoethanol, 10 mM Hepes (Sigma), and Gentamycin reagent solution (GIBCO/BRL), and stimulated with 10 nM $OVA_{323-339}$. After 7 days culture, $CD4^+$ T cell blasts in the culture were recovered by anti-CD4 Ab microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). BALB/c mice were immunized with an i.p. injection of 10 μg of DNP-BSA absorbed to 2 mg of aluminum hydroxide gel. After 3 wk, $Thy\text{-}1^-$ cells in the splenocytes were recovered by negative selection using anti-Thy-1 Ab microbeads (Miltenyi Biotech). The mixture of $CD4^+$ T cells (1×10^5 cells/well) and the $Thy\text{-}1^-$ cells (5×10^5 cells/well) in 200 μl of culture medium was restimulated with 10 ng/ml DNP-OVA in 96-well plates in the presence of a GIF sample. After 24 h, the cells were washed three times with and resuspended in the culture medium and then were cultured for 6 additional days. Anti-DNP IgE Abs in the culture supernatants were measured as described (13).

Inhibition of High Affinity Binding of GIF Derivatives to GIF Receptor. The affinity of GIF and its derivatives for the high affinity receptor on the 12H5 cells were compared by their ability to inhibit the binding of 1 nM ^{125}I-labeled C57A/N106S to the cells (20). Serial dilutions of a GIF sample to be tested were mixed with ^{125}I-labeled C57A/N106S, and each mixture was added to a cell suspension in duplicate. After incubation for 20 min at 37°C, cell-bound radioactivity was determined by the procedures described (20). Nonspecifically bound radioactivity was determined by the addition of 100-fold excess of unlabeled C57A/N106S to the system. Inhibition of the binding was determined by the ratio (specifically bound cpm in the presence of a sample)/(specifically bound cpm in the absence of the sample).

Fractionation of rhGIF Derivatives by Anti-HG3 Ab. A 10-μg GIF sample in 1 ml of PBS was mixed overnight at 4°C with 1 ml of anti-HG3 Ab-coupled HiTrap. After recovery of flow-through fraction (1 ml), the column was washed with 40 column volumes of PBS, and proteins retained in the column were eluted with 0.1 M glycine-HCl (pH 3.0).

Results

Biochemical Identification of Posttranslational Modifications of GIF in Ts Cells. GIF in the culture supernatants of the 31E9 cells was affinity-purified by using anti-GIF Ab-coupled HiTrap column. The purified GIF gave a single band of 13 kDa in SDS/PAGE and silver staining (Fig. 1*A*). MS analysis of the purified GIF revealed four M_r species of 12,346, 12,429, 12,467, and 12,551, respectively (Fig. 1*B*). The M_r of the smallest species was identical to the theoretical value calculated from the amino acid sequence of GIF which lacked the first methionine in the deduced sequence, and to the M_r of cytosolic GIF (13). Thus, the remaining three M_r species with higher M_r appear to be the molecules posttranslationally modified in 31E9 cells. As reported in

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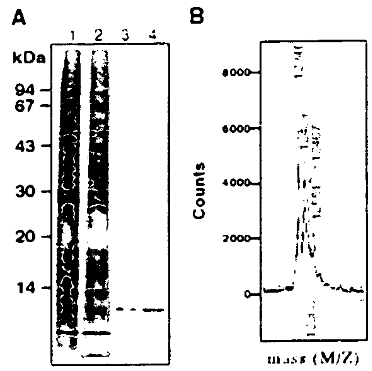

Fig. 1. Analysis of the GIF species in the culture supernatant of 31E9 cells. (*A*) The concentrated culture supernatant (lane 1), the effluent (lane 2) and acid eluate fraction (lane 3) from anti-GIF Ab-coupled HiTrap, and rhGIF (lane 4) were analyzed by SDS/PAGE and silver staining. (*B*) MS analysis of affinity-purified GIF from 31E9 cells. Numbers in the figure represent the M_r of each species.

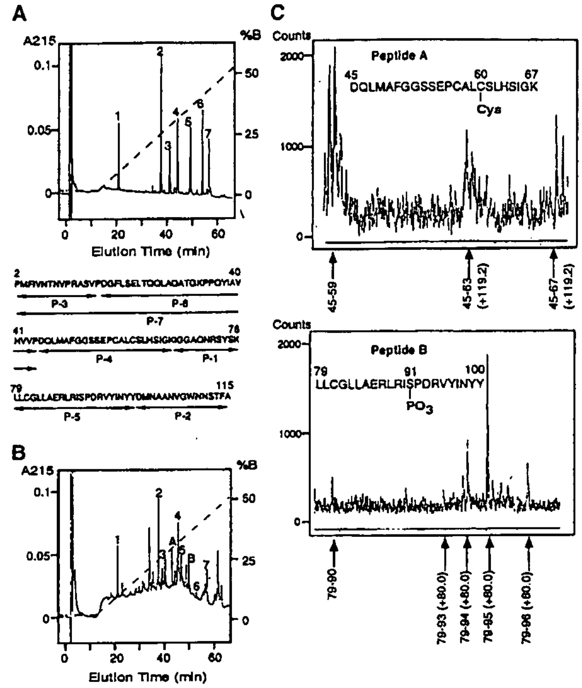

Fig. 2. Peptide map of rhGIF (*A*) and affinity-purified GIF from 31E9 cells (*B*). GIF were digested with API and AspN, and the digests were fractionated by RP chromatography. A dotted line indicates the percentage of solution B. Amino acid sequences of rhGIF-derived peptides is shown in *A*. The modified peptides were designated as peptide A and peptide B in *B*. (*C*) MS analysis of the peptides derived from peptide A (*Upper*) and peptide B (*Lower*); the peptides were obtained by digestion with carboxypeptidase Y.

a previous paper (13), inactivation of bioactive GIF from the 31E9 cells by the treatment with 1 mM DTT was accompanied by conversion of the 12,467 and 12,551 M_r species to the 12,346 and 12,429 M_r species, respectively. The results indicate that posttranslational modifications consist of two steps: covalent binding of a chemical group of M_r 83, and the binding of a group of M_r 121, the latter of which is susceptible to reducing reagent.

We have also examined physicochemical properties of GIF protein secreted from BUC cells, which were used as the fusion partner for establishing the Ts hybridoma, 31E9 cells. Affinity purification of the GIF protein, followed by MS analysis showed that the GIF protein secreted from BUC cells was homogeneous, and represented the 12,346 M_r species (data not shown).

To determine the posttranslational modifications, both the purified GIF from the 31E9 cells, described above, and rhGIF were digested with API and AspN, and the digests were fractionated by reverse-phase chromatography. The profile of the peptides from rhGIF and 31E9-derived GIF are shown in Fig. 2 *A* and *B*, respectively. The peptide map of the 31E9-derived GIF contained all seven peptides obtained from rhGIF. MS analysis of each of the seven peptides has shown that the peptides from the 31E9-derived GIF were identical to the peptides from rhGIF. However, comparisons between the peptide maps of rhGIF and the 31E9-derived GIF revealed that two peptides (peptide A and B) present in the digest of the latter preparation do not exist in the digests of rhGIF (Fig. 2*A* and 2*B*). The M_r of the peptide A and peptide B, determined by MS, were 2469.0 and 2719.5, respectively. These results, together with the amino acid sequence of the two peptides, indicated that peptide A represents peptide P-4 (M_r 2349.1) to which a chemical group of 119.9 Da bound, whereas peptide B represents peptide P-5 (M_r 2639.5), to which a chemical group of 80.0 Da covalently bound (compare Fig. 2*A*).

To determine which amino acids in the peptide A and B were modified, the peptides were digested with carboxypeptidase Y, and the fragments isolated by reverse-phase chromatography were analyzed by MS. The M_r of one fragment of peptide A, representing Asp-45 to His-63, was larger than that of the theoretical value by 119.2, but the M_r of another fragment, Asp-45 to Leu-59, was identical to that of the theoretical value (Fig. 2*C*). The results indicated that a chemical group of 119.2 Da must be covalently bound to one of the four amino acids from Cys-60 through His-63. Previous experiments have shown that the chemical group of 120 Da dissociated from GIF sequence by treatment with a reducing reagent (13), suggesting that the group bound to the peptide through a disulfide bond. Among the four amino acid residues, Cys-60 is only the amino acid that could form a disulfide bond. Thus, the results collectively indicate that the modification would be cysteinylation, or attachment of a second cysteine residue to Cys-60 via disulfide bond, which increases the M_r of the peptide by 120.

Similar analysis of the digest of peptide B displayed that the M_r of one fragment, Leu-79 to Asp-93 was larger than the theoretical value by 80.0 Da, whereas the M_r of another fragment, Leu-79 to Ile-90 was identical to that of the theoretical value (Fig. 2*C*). The results suggested that a chemical group of 80.0 Da is associated with one of the amino acid residues of Ser-91, Phe-92, and Asp-93. An increase in the M_r of 80.0 Da is consistent with phosphorylation. Among the three amino acid residues, only Ser-91 has the potential to be phosphorylated. Thus, we concluded that one of the chemical modifications of GIF in the Ts cells would be phosphorylation of Ser-91.

Immunosuppressive Activity of Cysteinylated GIF. The affinity-purified GIF from the culture supernatant of the 31E9 cells was fractionated by gel filtration through a Superdex 75 column. As shown in Fig. 3*A*, GIF could be fractionated into three peaks. MS analysis of each peak showed that peak 1 represented cysteinylated GIF (M_r 12,467), whereas peak 2 and 3 were mixtures of phosphorylated GIF (M_r 12,429) and unmodified GIF (M_r 12,346). Approximately 30% of the total GIF in a pool of peak 2 and 3 was phosphorylated GIF, and the remainder was unmodified GIF. Because the concentration of the 12,551 M_r species in the original GIF preparation was too low, (lower than that observed in Fig. 1*C*), this species could not be isolated in this fractionation.

Experiments were carried out to determine immunosuppres-

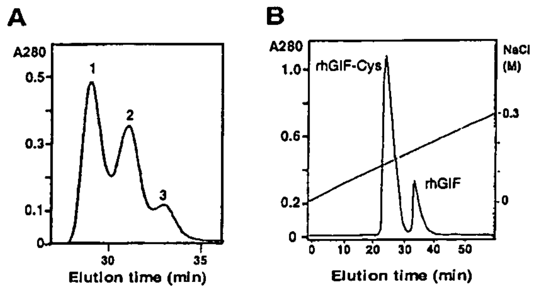

Fig. 3. (*A*) Gel filtration of affinity-purified GIF through a Superdex 75 column. Main protein peaks were numbered. (*B*) Fractionation of cysteinylated rhGIF on a CM-5PW column. Linear line indicates the gradient of NaCl.

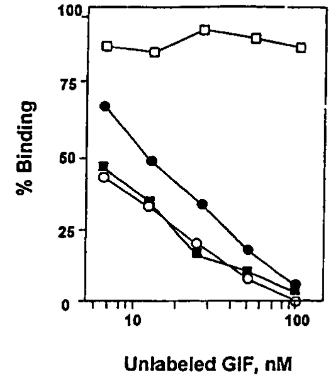

Fig. 5. Inhibition of the binding of ^{125}I-labeled C57A/N106S to 12H5 cells by cysteinylated GIF proteins. Radiolabeled C57A/N106S was mixed with various concentrations of rhGIF (□), C57A/N106S (○), nGIF-Cys (●), or rhGIF-Cys (■), and the mixtures were incubated with 1×10^6 cells. The final concentration of ^{125}I-labeled C57A/N106S was 1 nM. The ordinate represents the ratio between specifically bound radioactivity in the presence of unlabeled GIF and that in the absence of GIF.

sive activities of posttranslationally modified GIF species. In view of previous findings that high affinity receptors for bioactive GIF are expressed on both activated Th cells and Ag-primed B cells, but not on naive T and B cells nor on macrophages (21), we determined the immunosuppressive effect of GIF on the secondary IgE Ab response of Ag-primed B cells to DNP-OVA in the presence of Ag-primed T cells from OVA-specific TCR$\alpha\beta$ transgenic mice (see *Materials and Methods*). Determination of immunosuppressive activities of peak 1 fraction and the pool of peak 2 and 3 on the *in vitro* IgE Ab response clearly showed that cysteinylated GIF (peak 1) suppressed the IgE Ab response, whereas the mixture of phosphorylated GIF and unmodified species failed to do so, even though the latter fraction contained twice as much GIF protein than the peak 1 fraction (Fig. 4).

To confirm the critical role of cysteinylation at Cys-60 for the generation of immunosuppressive activity, attempts were made to prepare a cysteinylated derivative of rhGIF. The wild-type rhGIF was treated with cystine following the procedure described in *Materials and Methods*, and the product was fractionated on a CM-5PW column. MS analysis of the two fractions shown in Fig. 3*B* indicated that the first fraction is the cysteinylated derivative (M_r 12,467), whereas the second peak represents unmodified rhGIF. The cysteinylated rhGIF in the first fraction was further purified by gel filtration through a Superdex 75 column. Unmodified rhGIF was not detectable in the final preparation by MS.

To confirm that Cys-60 in rhGIF was selectively cysteinylated, the cysteinylated rhGIF was digested with API and AspN, and the digest was subjected to peptide mapping. The profile of the peptides was essentially the same as that of 31E9-derived GIF (compare Fig. 2*B*), except that peptide B was not detectable.

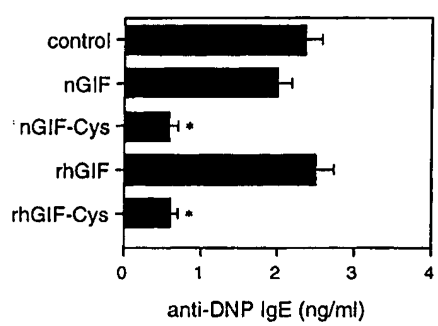

Fig. 4. Suppression of *in vitro* IgE Ab response by cysteinylated GIF from 31E9 cells [native (n) GIF-Cys] and cysteinylated derivative of rhGIF (rhGIF-Cys). The culture contained 1 μg/ml of GIF from a pool of peak 2 and 3 in Fig. 3 (nGIF); 500 ng/ml of GIF from peak 1 (nGIF-Cys); 500 ng/ml rhGIF, or 500 ng/ml rhGIF-Cys. The figure shows the concentration of anti-DNP IgE Abs in culture supernatants. Each bar is the mean ± SEM of 10 wells. *, $P < 0.05$.

Digestion of the peptide A from the cysteinylated rhGIF with carboxypeptidase Y, followed by MS analysis of the fragments confirmed that Cys-60 in the sequence was cysteinylated. Thus, we determined immunosuppressive activity of the cysteinylated rhGIF on the anti-DNP Ab response. As shown in Fig. 4, the immunosuppressive activity of the cysteinylated rhGIF was comparable to that of the cysteinylated GIF isolated from the culture supernatant of the 31E9 cells.

Binding Capacity of Cysteinylated GIF Derivatives to Target Cells. Previous experiments have shown that bioactive GIF from Ts cells and bioactive derivatives of rhGIF bind to the receptors on Th hybridomas, activated T and B cells with high affinity, whereas inactive, cytosolic GIF and *E. coli*-derived wild-type rhGIF failed to do so (20, 21). High affinity binding capacity of GIF molecules for the target cells was generated by replacement of Cys-57 with Ala, and of Asn-106 with Ser or by binding of 5-thio(2-nitrobenzoic acid) group to Cys-60 in the C57A molecules. The K_d of the specific binding between the high affinity receptors on the Th hybridoma, 12H5 cells and bioactive rhGIF derivatives, such as C57A/N106S and C57A-DTNB, was in the 10–100 pM range (20).

Because the bioactive GIF species in the culture supernatant of 31E9 cells appears to be cysteinylated GIF, we determined the ability of the 31E9-derived cysteinylated GIF and cysteinylated rhGIF to inhibit the binding of ^{125}I-labeled C57A/N106S to the 12H5 cells. The results of the experiments are shown in Fig. 5. As expected, wild-type rhGIF failed to inhibit the binding of the radiolabeled C57A/N106S even with 100-fold excess, but both the 31E9-derived cysteinylated GIF and cysteinylated rhGIF inhibited the binding in a dose-dependent manner. It should be noted that the ability of the cysteinylated rhGIF to inhibit the binding of ^{125}I-labeled C57A/N106S was comparable to that of the homologous ligand. Conversely, C57A/N106S inhibited the specific binding of ^{125}I-labeled cysteinylated rhGIF to 12H5 cells as effective as unlabeled cysteinylated rhGIF (data not shown).

Evidence for Conformational Changes in GIF Molecules Modified by Cysteinylation. The present results indicate that bioactivity of GIF is generated by the binding of cysteine to Cys-60. Previous findings also indicated that a weak bioactivity was generated by carboxymethylation of Cys-60 (12) or replacement of Cys-57 with Ala (13), and when combined the two chemical changes had a synergistic effect on increasing the bioactivity and the affinity

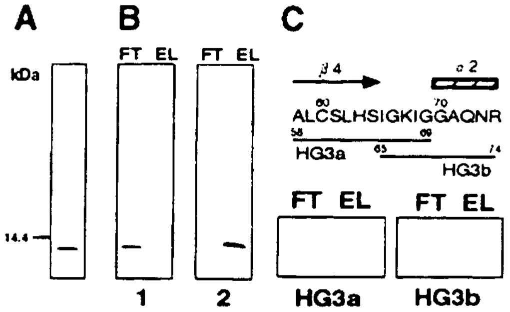

Fig. 6. Specific binding of cysteinylated rhGIF derivatives to anti-HG3. (*A*) Immunoblotting of rhGIF with anti-HG3 Abs. (*B*) rhGIF (1) or cysteinylated rhGIF (2) were fractionated on anti-HG3 Abs-coupled HiTrap, and the flow through (FT) and acid-eluate (EL) fractions were analyzed by immunoblotting with anti-GIF. (*C*) A 10-μg aliquot of cysteinylated rhGIF was mixed with 1 mg/ml of either HG3a or HG3b peptide, and the mixture was fractionated on anti-HG3-coupled column. The fractions were analyzed by immunoblotting with anti-GIF. Amino acid sequences of HG3a and HG3b peptides are shown above the immunoblot.

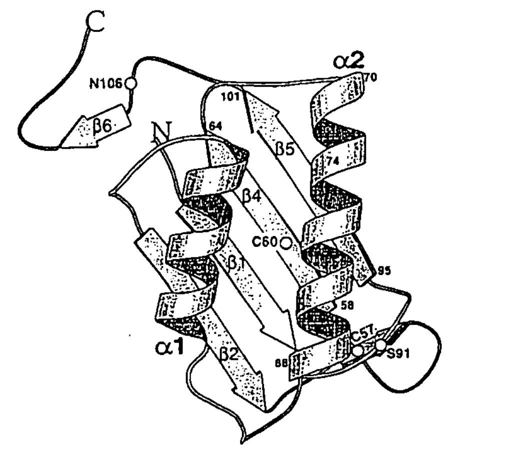

Fig. 7. Ribbon diagram of GIF monomer. Numbers represent the position of amino acid residues.

of the molecules for the target cells (20). One of the possible explanation for why such disparate modifications result in an increase in bioactivity is that they alter the conformation of GIF such that it is converted to an active structure. X-ray crystal structure of rhGIF suggested high conformational flexibility in the α helices and adjacent loop region in the rhGIF (22) (Fig. 7). We wondered whether cysteinylation of Cys-60 might have induced conformational changes in these regions. To test this possibility, we prepared polyclonal Abs against the peptide region Ala-58–Arg-74 (HG3), which forms the β4 strand, loop, and amino terminal one-third of the α2 helix in rhGIF. SDS-PAGE of rhGIF, followed by immunoblotting with the specifically purified anti-HG3, clearly showed that the Abs bound to the 13-kDa GIF under these experimental conditions (Fig. 6*A*). However, fractionation of the wild-type rhGIF and cysteinylated rhGIF on the anti-HG3-coupled immunosorbent showed that rhGIF failed to be retained in the column. In contrast, cysteinylated rhGIF bound to the column and was recovered by elution at acid pH (Fig. 6*B*). We also have found that C57A-DTNB, a highly bioactive derivative of rhGIF, in which Cys-57 was replaced with Ala and 5-thiobis(2-nitrobenzoic acid) group was bound to Cys-60 (12), bound to anti-HG3 column and was recovered by acid elution (data not shown). The results suggested that the epitope(s) recognized by the Abs are hidden in the wild-type GIF molecules but exposed by the modifications that result in the bioactive GIF derivatives.

Because the peptide used for the preparation of the Abs covers the amino acid sequence for the β4 strand and α2 helix in rhGIF (Fig. 6*C*), attempts were made to further map the epitope recognized by the Abs. Thus, cysteinylated rhGIF was fractionated on the anti-HG3-coupled column in the presence of a synthetic peptide, HG3a, which covers the β4 strand and loop region, or peptide HG3b, which covers the loop region and α2 helix in the original rhGIF molecules. The results shown in Fig. 6*C* indicated that HG3a did not inhibit the binding of the cysteinylated rhGIF to the immunosorbent, whereas the peptide HG3b completely inhibited the binding. These findings do not exclude the possibility that the Ab preparation contains Abs against HG3a, but simply indicate that such Abs, if they exist, do not bind either rhGIF or the cysteinylated derivative. In any event, binding of the Abs specific for HG3b to cysteinylated rhGIF, but not to the wild-type rhGIF, indicates that certain critical amino acid residues in the region of the HG3b peptide are exposed to the solvent face of the cysteinylated rhGIF and capable of binding the anti-HG3 Abs, but the epitope is hidden in the wild-type rhGIF. These results strongly suggest that cysteinylation of Cys-60 causes a conformational change in the α2 helix region (Fig. 7).

Discussion

In the present experiments, we have identified the posttranslational modifications of cytosolic GIF/MIF molecules in the Ts hybridoma 31E9 cells as cysteinylation of Cys-60 and phosphorylation of Ser-91. The two independent processes of posttranslational modification explain the presence of the four M_rs, i.e., 12,551, 12,467, 12,429, and 12,346, species in the culture supernatant of Ts cells. The proportion of the four species in the supernatant depends on the culture conditions and cell lines used. Because culture media for the 31E9 cells contained 0.2 mM cystine, the possibility may be considered that cysteinylation of GIF took place by a disulfide exchange reaction after the wild-type GIF was released from the cells. However, this possibility is quite unlikely, because culture supernatants of BUC cells, a fusion partner for establishing the hybridoma, contained only the 12,346 M_r species. The results are in accord with the fact that the GIF protein in the culture supernatant of BUC cells is inactive (10) and indicate that posttranslational modification of GIF is unique for Ts cells. In view of the fact that cysteinylation and phosphorylation of peptides occur in subcellular organelles (23), one may speculate that Ts cells may have a unique transporter protein, which facilitates translocation of the GIF peptide to subcellular organelles where the posttranslational modifications of the peptide take place.

The present experiments showed that cysteinylation of Cys-60 is responsible for the generation of bioactivity. The conclusion was supported by the fact that bioactivity of the cysteinylated rhGIF, in which phosphorylation of Ser-91 is lacking, was as active as the cysteinylated GIF from Ts hybridoma. As we were not able to isolate the phosphorylated GIF in the present experiment, one cannot exclude a possible role of phosphorylation of Ser-91 in the generation of bioactivity. However, this possibility is unlikely because a mixture of phosphorylated GIF and unmodified GIF failed to show any immunosuppressive effect. Furthermore, previous experiments showed that treatment of partially purified GIF from culture supernatants of Ts cells with alkaline phosphatase resulted in an increase in GIF bioactivity of the preparations (5). Because GIF in culture supernatants of Ts hybridomas contain the 12,551 M_r species in which both Cys-60 and Ser-91 are modified, the enhancement of bioactivity by the treatment with alkaline phosphatase would be due to dephosphorylation of Ser-91 in this species to form

cysteinylated GIF. Thus, these results suggest that phosphorylation of Ser-91, if it has any function, either diminishes or inactivates cysteinylated GIF.

The GIF molecule contains three cysteine residues (Cys-57, -60, and -81) (6). Upon posttranslational modification, however, cysteine binds only to Cys-60, but neither to Cys-57 nor to Cys-81. This finding is in agreement with the fact that treatment of rhGIF with cystine resulted in selective cysteinylation of Cys-60. Previous experiments showed that the treatment of rhGIF with DTNB resulted in the binding of 5-thiobis(2-nitorobenzoic acid) to Cys-60, but not to the other two cysteine residues. Treatment of rhGIF with iodoacetate also resulted in selective carboxylmethylation of Cys-60 (12).

However, the generation of bioactivity does not appear to be due merely to loss of the free sulfhydryl group of Cys-60. Binding of cysteine or 5-thiobis(2-nitorobenzoic acid) group to Cys-60 in rhGIF through a disulfide bond resulted in the formation of derivatives, which are comparable in the bioactivity to the Ts-derived GIF, but carboxylmethylated GIF was 20-fold less active (12). It also was found that the bioactivity of the derivatives did not directly correlate with the size of the chemical group bound to the SH group of Cys-60. In an attempt to prepare stable bioactive derivatives of rhGIF, we coupled several compounds, such as α-bromophenylacetic acid and 2-bromohexanoic acid, to Cys-60 of rhGIF, which increased the M_r by 122 Da and 114 Da, respectively. However, coupling of such compound to Cys-60 failed to generate the bioactivity (results not shown).

Because the chemical group bound to the sulfhydryl group of Cys-60, which would protrude between the two α helices (cf. Fig. 7), we speculate that charged group(s) in cysteine or 5-thiobis(2-nitorobenzoic acid) group may interact with amino acid residue(s) in an α helix, and causes conformational changes in the helix. This speculation is supported by the present experiments, which showed that polyclonal Abs specific for a stretch of amino acid sequence in the $\alpha 2$ helix bound to cysteinylated GIF but failed to bind wild-type rhGIF. As the stretch of amino acids, i.e., Gly-70–Arg-74 form a part of the helical structure in wild-type rhGIF (see Fig. 7), the complete epitope would not be exposed to the solvent face of the molecules. Binding of the Abs to cysteinylated GIF suggests unfolding of this portion of the helical structure in cysteinylated GIF. Similar approaches using Abs for the detection of conformational changes in protein molecules have been described by other investigators (24, 25).

The present experiment showed that cysteinylation of GIF/MIF protein, which occurred during the posttranslational modification process in Ts cells, and consequent conformational changes in the protein molecules conferred a unique structure that has affinity for the GIF receptors on activated T and B cells. The wild-type rhGIF, which is identical to MIF, lacked affinity for the receptors on activated T and B cells (21), and therefore failed to affect the Ab response (see Figs. 4 and 5). On the other hand, MIF has been shown to be a proinflammatory cytokine. The recombinant mouse MIF was reported to induce secretion of tumor necrosis factor-α from RAW264.7 macrophage line cells (26). However, cysteinylated rhGIF (or bioactive GIF) lacked affinity for the cell line cells and failed to induce tumor necrosis factor-α (20). Thus, although it is obvious that MIF and GIF share an identical structure gene, a series of our experiments, including those described in the present report, indicate that cysteinylation of GIF/MIF during the process of posttranslational modifications in Ts cells induces conformational structural changes in the molecules, which drastically changes their biologic functions. Although the relationship between GIF and MIF may be somewhat unique, these findings call attention to the possibility that posttranslational modifications of other bioactive cytokines may regulate their function.

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1. Ishizaka, K. (1984) *Annu. Rev. Immunol.* **2,** 159–182.
2. Jardieu, P., Uede, T. & Ishizaka, K. (1984) *J. Immunol.* **133,** 3266–3273.
3. Nakano, T., Ishii, Y. & Ishizaka, K. (1996) *J. Immunol.* **156,** 1728–1734.
4. Ishii, Y., Nakano, T. & Ishizaka, K. (1996) *J. Immunol.* **156,** 1735–1742.
5. Akasaki, M., Jardieu, P. & Ishizaka, K. (1986) *J. Immunol.* **136,** 3172–3179.
6. Mikayama, T., Nakano, T., Gomi, H., Nakagawa, Y., Liu, Y.-C., Sato, M., Iwamatsu, A., Ishii, Y., Weiser, W. Y. & Ishizaka, K. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10056–10060.
7. Weiser, W. Y., Temple, P. A., Witek-Giannotti, J. S., Remold, H. G., Clark, S. C. & David, J. R. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 7522–7526.
8. Paralkar, V. & Wistow, G. (1994) *Genomics* **19,** 48–51.
9. Bernhagen, J., Mitchell, R. A., Calandra, T., Voelter, W., Cerami, A. & Bucala, R. (1994) *Biochemistry* **33,** 14144–14155.
10. Liu, Y.-C., Nakano, T., Elly, C. & Ishizaka, K. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 11227–11231.
11. Nakano, T., Liu, Y.-C., Mikayama, T., Watarai, H., Taniguchi, M. & Ishizaka, K. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 9196–9200.
12. Nakano, T., Watarai, H., Liu, Y.-C., Oyama, Y., Mikayama, T. & Ishizaka, K. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 202–207.
13. Tomura, T., Watarai, H., Honma, N., Sato, M., Iwamatsu, A., Kato, Y., Kuroki, R., Nakano, T., Mikayama, T. & Ishizaka, K. (1999) *J. Immunol.* **162,** 195–202.
14. Huff, T. F. & Ishizaka, K. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 1514–1518.
15. Iwata, M., Adachi, M. & Ishizaka, K. (1988) *J. Immunol.* **140,** 2534–2542.
16. Sato, T., Sasahara, T., Nakamura, Y., Osaki, T., Hasegawa, T., Tadakuma, T., Arata, Y., Kumagai, Y., Katsuki, M. & Habu, S. (1994) *Eur. J. Immunol.* **24,** 1512–1516.
17. Sette, A., Buss, S., Colon, S., Miles, C. & Grey, H. M. (1988) *J. Immunol.* **141,** 45–48.
18. Liu, F. T., Bohn, J. W., Ferry, E. L., Yamamoto, H., Molinaro, C. A., Sherman, L. A., Klinman, N. R. & Katz, D. H. (1980) *J. Immunol.* **124,** 2728–2737.
19. Hirano, T., Miyajima, H., Kitagawa, H., Watanabe, N., Azuma, M., Taniguchi, O., Hashimoto, H., Hirose, S., Yagita, H., Furusawa, S., *et al.* (1988) *Int. Arch. Allergy Appl. Immunol.* **85,** 47–54.
20. Sugie, K., Nakano, T., Tomura, T., Takakura, K., Mikayama, T. & Ishizaka, K. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 5278–5283.
21. Sugie, K., Tomura, T., Takakura, K., Kawano, T., Taniguchi, M. & Ishizaka, K. (1999) *Int. Immunol.* **11,** 1149–1156.
22. Kato, Y., Muto, T., Tomura, T., Tsumura, H., Watarai, H., Mikayama, T., Ishizaka, K. & Kuroki, R. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 3007–3010.
23. Hwang, C., Sinskey, A. J. & Lodish, H. F. (1992) *Science* **257,** 1496–1502.
24. Yu, Y. Y. L., Myers, N. B., Hilbert, C. M., Harris, M. R., Balendiran, G. K. & Hansen, T. H. (1999) *Int. Immunol.* **11,** 1897–1905.
25. Wagner, B. L., Bauer, A., Schutz, G. & Montminy, M. (2000) *J. Biol. Chem.* **275,** 8263–8266.
26. Calandra, T., Bernhagen, J., Mitchell, R. A. & Bucala, R. (1994) *J. Exp. Med.* **179,** 1895–1902.